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Validation of the nickel biotic ligand model for locally relevant species in Australian freshwaters

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Abstract

Australian freshwaters have relatively low water hardness and different calcium to magnesium ratios compared with those in Europe. The hardness values of a substantial proportion of Australian freshwaters fall below the application boundary of the existing European nickel Biotic Ligand Models (NiBLMs) of 2 mg Ca/L. Toxicity testing was undertaken using *Hydra viridissima* to assess the predictive ability of the existing NiBLM for this species in extremely soft waters. This testing revealed a greater effect of calcium and magnesium in competing with nickel for binding to the biotic ligand in soft water (<10 mg $CaCO_3$/L) than at higher water hardness. Modifications were made to the NiBLM to account for softer waters encountered in Australia and the more important competitive effect of calcium and magnesium on nickel toxicity. To validate the modified NiBLM, ecotoxicity testing was performed on five Australian test species in five different natural Australian waters. Overall, no single water chemistry parameter was able to indicate the trends in toxicity to all of the test species. The modified NiBLMs were able to predict the toxicity of nickel to the test species in the validation studies in natural waters better than the existing NiBLMs. This work suggests that the overarching mechanisms defining nickel bioavailability to freshwater species are globally similar, and that NiBLMs can be used in all freshwater systems with minor modifications.

Keywords

bioavailability, biotic ligand model, nickel, tropical ecotoxicology

Introduction

Accounting for bioavailability provides an ecologically and environmentally relevant metric by which to assess potential risks from metals to aquatic communities. Using bioavailability in a regulatory context provides an evidence-based way to assess compliance and, importantly, to prioritize and rank locations which are potentially at risk (Merrington et al. 2016). The OECD recently concluded that bioavailability should be taken into account for assessing the environmental risk of metals, and for setting environmental quality standards for metals (OECD 2016).

Chronic BLMs for nickel (Ni) have been developed for *Pseudokirschneriella subcapitata* (Deleebeeck et al. 2009), *Daphnia magna* (Deleebeeck et al. 2008), *Ceriodaphnia dubia* (de Schamphelaere et al. 2006), and *Oncorhynchus mykiss* (Deleebeeck et al. 2007). These nickel BLMs have been combined with an ecotoxicity database to produce a bioavailability normalized species sensitivity distribution (SSD) (Nys et al. 2016). This nickel BLM (NiBLM) has been adopted as the basis of the environmental quality standard (EQS) for nickel in Europe to enable site-specific EQS values to be derived (EC 2010). The NiBLM applies species-specific BLMs across broad taxonomic groups (i.e. vertebrates, invertebrates, plants) to normalize all of the ecotoxicity data to calculate a site-specific EQS or water quality guideline value from a bioavailability normalized SSD.

Based on the current global trend of basing EQSs for metals on a bioavailability-normalized basis, considerable value can be gained by using bioavailability models in revisions to the Australian and New Zealand water quality guideline values for nickel. The purpose of this

68 study was to evaluate the validity of currently available nickel bioavailability models and
69 normalization approaches (Nys et al. 2016) for Australian freshwaters and Australian aquatic
70 species.

71 The European regulatory requirement to apply slightly different bioavailability models to each
72 trophic level has resulted in a number of models that have little difference between them, and
73 are approximately equivalent in their ability to predict nickel bioavailability to relevant species.
74 The principal difference between the different biotic ligand models that are applied to chronic
75 nickel toxicity to individual species is how they handle pH effects, and how these effects are
76 parameterized for the different species. The empirical approach taken was originally
77 developed because the mechanistic approach used for the *P. subcapitata* BLM was unable to
78 explain the effects of pH over a sufficiently broad range of conditions. The *P. subcapitata* BLM
79 (Deleebeeck et al. 2009) assumes that H^+ competes directly with Ca^{2+}, Mg^{2+}, and Ni^{2+} for
80 occupancy of the biotic ligand. An alternative, empirical, approach is applied in the BLMs for
81 *D. magna* (Deleebeeck et al. 2008), *C. dubia* (de Schamphelaere et al. 2006), and *O. mykiss*
82 (Deleebeeck et al. 2007), which handles pH effects separately from the competitive
83 interactions between divalent cations at the biotic ligand.

84 The principal goals of this research were two-fold:

85 1. To determine if the ranges of freshwater chemical parameters for which the existing
86 BLMs were developed are similar to the ranges in Australia, which would indicate that
87 the models were applicable from the chemical perspective; and

88 2. To determine if the relationships between chronic toxicity and physico-chemical water
89 characteristics developed for three trophic levels of European standard species are
90 similar for species from a different geographic region, i.e. Australia.

Water hardness (calcium and magnesium concentrations), pH, and dissolved organic carbon (DOC) are key parameters for existing NiBLMs. The distributions of these parameters in Australian waters needs to be established to determine the relevance of the existing developed NiBLMs to Australian waters. The assumption is that the relationships between chronic toxicity and physico-chemical water characteristics upon which the NiBLM is based are broadly consistent across different species and continents. A two-fold approach was taken to test this assumption. First, geochemical parameters were collected to test if the ranges for which the models were originally developed are relevant to Australia (Nys et al. 2016). Second, toxicity tests were performed in natural Australian waters with different water chemistry and nickel bioavailability using Australian test species, to allow comparisons with previously developed models. Australia provides a good test of the transferability of the BLM concepts due to the fact that some resident species have evolved in relative isolation from European and North American species which are most frequently represented in existing ecotoxicity databases.

Methods

Geochemistry of Australian Waters. A steering group of Australian and New Zealand regulatory scientists, researchers, consultants and industry representatives identified fresh surface water datasets that were held by contacts in Australian State and Territory regulatory organisations.

There were 11,744 samples from a total of 1465 sites in the dataset, although not all of the sites had full coverage of all of the required physico-chemical parameters (pH, DOC, Ca and Mg). There were 264 sites from New South Wales, 204 sites from the Northern Territory, 42 from Queensland, 8 from South Australia, 870 from Tasmania, 66 from Victoria and 1 from Western Australia, plus 5 samples from unknown locations.

Model Performance Testing in Very Soft Waters. *Hydra viridissima*, a tropical green cnidarian which is native to the soft water conditions in Northern Australia, was used to assess the chronic toxicity of nickel in eight different synthetic waters with varying water hardness, and calcium and magnesium ratios, according to the methods outlined by Riethmuller et al. (2003). Hardness in these test waters ranged between 1.1 and 54 mg $CaCO_3$/L in order to assess whether or not the BLM would be applicable to extremely soft waters such as Magela Creek which has been used as a reference water for toxicity testing in Australia (Harford et al. 2015). Both calcium and magnesium concentrations in the softest five test waters were below 2 mg/L, and the softest four test waters all had water hardness values below 5 mg $CaCO_3$/L. By contrast, the lower validation limit of the current NiBLM used in Europe is 2 mg Ca/L (hardness ~8 mg $CaCO_3$/L).

Testing was performed at a range of Ca:Mg ratios (0.3 to 2.1 mol Ca:Mg), which were chosen to reflect those typical of Australian and European waters. Magnesium concentrations were adjusted according to the calcium concentration, based on empirical relationships established from European (Peters et al. 2011) or Australian (Supporting Information) freshwaters (Figure 1 and Table 2).

Toxicity tests on *H. viridissima* were performed by the Environmental Research Institute of the Supervising Scientist in Darwin, Australia, according to standard protocols assessing population growth over 96 h, with daily feeding and renewal of test solutions (full details available in Supporting Information). Briefly, the toxicity tests were performed at 27±1°C and pH 6.5±0.1 in amended synthetic soft water (SSW) to increase the concentrations of calcium and magnesium from the baseline conditions (hardness of 1 mg $CaCO_3$/L) in the softest tested water. Test waters were made up 48h prior to the start of toxicity tests to allow for pH equilibration. Exposure concentrations were based on the average measured concentrations in fresh and 24-h old exposure media. Non-linear regression (3-parameter log-logistic)

analyses were used to determine point estimates of inhibitory concentrations (ICs) that reduced growth rate by 10% and 50% (i.e. IC10 and IC50) relative to the synthetic softwater control responses (CETIS v1.8.1.2, Tidepool Scientific Software).

Model Validation Testing. Toxicity testing was performed on five different Australian species (including four tropical, soft water resident species, Harford et al. 2015) in five different field-collected Australian freshwaters. The test species are all routinely used for toxicity testing in Australia and each species represents a different taxonomic group. The tested species were the rainbowfish *Melanotaenia splendida splendida*, the cladoceran *Ceriodaphnia dubia*, the green hydra *Hydra viridissima*, the duckweed *Lemna aequinoctialis*, and the green alga *Chlorella* sp.. *C. dubia* is neither tropical nor is it resident in very soft waters. The test waters were selected to maximize the range of water chemistry parameters likely to be observed throughout Australia, and hence to provide a range of nickel bioavailabilities. Therefore, the chemistry of each selected water differed in some respects from each of the other test waters (Table 1). The water chemistry in the tests performed in the field-collected waters differed slightly between different species for the same water (see paragraph below). Measured values were used for all BLM calculations and in some cases these differed slightly from the values reported in Table 1, the conditions for each test can be found in the supporting information.

Toxicity tests on *Chlorella* sp. were performed on samples collected independently from those used for other species, resulting in some minor differences in the water chemistry for these tests. Similarly, the presence of the test organisms had small but varying effects on the pH of the test waters. Average (mean) values are reported for the water chemistries in Table 1, although the measured values were used for modelling purposes (see supporting information).

Toxicity tests with the green unicellular freshwater algae, *Chlorella* sp., were undertaken in accordance with the methodology described by Franklin et al. (1998) performed at 29±1°C, no EDTA was added to the test waters. Exponentially growing cells from a 4 to 5-d old culture and at an initial algal cell density of 3 x 10^4 cells/mL were exposed to at least 5 concentrations of nickel (each in triplicate) over a 72-h period. The test method involved counting cells at 24, 48 and 72 h by flow cytometry in order to calculate daily growth rates.

The chronic (partial life-cycle) toxicity tests with the freshwater cladoceran *C. dubia* were undertaken according to USEPA (2012) and adapted for use with the locally collected *C. dubia* (referred to as the Sydney clone (Bailey et al. 2000)). The freshwater tropical aquatic duckweed test using *L. aequinoctialis* was undertaken in accordance with Reithmuller et al. (2003). This procedure is based on OECD method 221 (15), but modified to a test temperature of 29±2ºC, and a duration of 96 h. For the freshwater green hydra population growth test using *H. viridissima*, the method set out in Reithmuller et al. (2003).

The rainbowfish embryo hatching test using *M. splendida splendida* was undertaken in accordance with Reithmuller et al. (2003). This procedure is based on the methodology described by the USEPA (2012) with the following exceptions: 1) the rainbowfish *M. splendida splendida* was used instead of the fathead minnow *Pimephales promelas*; 2) a fish imbalance endpoint was used as a surrogate for the mortality endpoint, which could not be used in the present study due to animal ethics restrictions; 3) the test duration was extended to 96 h to accommodate the longer hatching time of *M. splendida splendida*; and, 4) five embryos were introduced into each replicate at the beginning of the test instead of ten, also due to animal ethics restrictions.

The IC/EC10 and IC/EC50 estimates from the tests were determined using the linear interpolation method using TOXCALC v5.0 or CETIS v1.8 (Tidepool Scientific Software).

Chemical Analysis

189 Field collected water samples for validation testing were analysed for a suite of trace elements,
190 BTEX, hydrocarbons, PAHs, organochlorine pesticides, and organophosphate pesticides in
191 order to ensure that they did not contain any potential toxicants which might interfere with
192 the results of toxicity tests with Ni.

193 At the start of each toxicity test filtered sub-samples (40 mL) of the control water, a procedural
194 blank and an ultra-pure water blank were collected in plastic sample vials and acidified with
195 1% Nitric acid (HNO_3). These samples were part of a quality control elemental suite used to
196 ensure that no unwanted elements were introduced into the test system. 14 mL filtered sub-
197 samples were also taken of each of the nickel treatments at the start and end of each test in
198 order to verify the dissolved (<0.45 µm) nickel concentrations and establish whether any
199 nickel had been lost from solution during the test period. Measured nickel concentrations were
200 used for statistical analyses. Verification of nutrient concentrations (nitrate and phosphate)
201 for *Chlorella* sp. and *L. aequinoctialis* tests were performed by taking a 50 mL sub-sample
202 from the control treatment and an ultra-pure water blank. These samples were frozen prior
203 to transportation for analysis. Samples were analysed at Envirolab Services Pty Ltd
204 (Chatswood, NSW) using ICP-MS or ICP-OES.

205

206 **Nickel Toxicity Modelling.**

207 The BLM fitting for the optimisation of binding constants under extremely soft water conditions
208 was performed using EC50 data only due to the increased reliability of this endpoint relative
209 to the EC10 data. The BLM fitting for species sensitivity was performed by calculating the
210 critical degree of accumulation of nickel at the biotic ligand at the specified effect
211 concentration (either the EC10 or the EC50) in each test. Chemical speciation calculations
212 were performed using WHAM 6 (NERC 2001, V 6.0.13), assuming that the activities of Fe^{3+}
213 and Al^{3+} were controlled by the solubility of their colloidal precipitates, with the concentration

of active fulvic acid assumed to be 0.8 times the DOC concentration, with the Ni-fulvic acid binding constant log K_{MA} set to 1.75, and using updated stability constants for the formation of nickel carbonate complexes as recommended by Van Laer et al. (2006). The critical nickel accumulation values were calculated based on the minimum sum of log-transformed errors between the calculated and observed results, and were averaged across all of the tests for the same species and endpoint in order to derive the corresponding fitted sensitivity parameter. Assessment of model parameters was based on EC50 values. No observed effect concentration (NOEC) values were not used for modelling, although they have been used as the critical endpoint in the development of some BLMs (Nys et al. 2016, van Sprang et al. 2009). Due to the relatively high similarity between the four NiBLMs available for different species (Deleebeeck et al. 2009, Deleebeeck et al. 2008, Schamphelaere et al. 2006, Deleebeeck et al. 2007), and the fact that the different models were a regulatory rather than a scientific requirement, a limited selection of the models was used to fit the toxicity data for each test species by adjusting the sensitivity parameters.

The *P. subcapitata* BLM (Deleebeeck et al. 2009) was applied for model fitting in the first instance because this model assumes direct competition between cations at the biotic ligand. The ability of the alternative models to provide improved fits to the experimental data was used to provide some insight into the possible differences in pH related responses to nickel toxicity between different organisms.

Results

Geochemistry of Australian Waters.

The median pH value of the Australian surface waters included in the database was approximately 6.9, and the 5th and 95th percentiles of the dataset were 5.5 and 8.2 respectively

(Table 2). The median DOC concentration was approximately 3.8 mg/L, and the 5th and 95th percentiles of the dataset were 1.5 mg/L and 17 mg/L respectively. The median calcium concentration was approximately 2.2 mg/L, and the 5th and 95th percentiles of the dataset were 0.2 mg/L and 84 mg/L respectively. The median magnesium concentration was approximately 1.8 mg/L, and the 5th and 95th percentiles of the dataset were 0.3 mg/L and 54 mg/L respectively.

A linear relationship was observed between $\log_{10}$(Ca) and $\log_{10}$(Mg) for 1816 samples with measured data for both parameters (Figure 1).

Toxicity Testing in Very Soft Waters. Toxicity tests performed on *H. viridissima* demonstrated a clear trend of increasing tolerance to nickel with increasing water hardness (Figure 2). The relationship between hardness and effect concentration was more precise when EC50 data were used, compared with relationships based on EC10 data. The tests were split into groups of harder and softer waters depending upon whether the hardness was above or below 5 mg/L $CaCO_3$ prior to following the approach used to determine the stability constants for the binding of competing ions according to de Schamphelaere et. al. (2002), see Table 4. The stability constants which would be calculated following this approach for the softer waters are higher than those which would be calculated for the harder waters. Increasing the log K values used for Ca and Mg to the biotic ligand from a value of 3.5 as used by the original models to a value of 5 reduced the rmse in the prediction of EC50 values from 135 to a value of 22.4. This effectively changes the relative binding affinities of Ca and Mg to Ni, meaning that Ca and Mg compete more effectively against Ni when binding to the biotic ligand in very soft waters. A version of the BLM with the log K values for both Ca and Mg adjusted to 5.0 based on this analysis is referred to as the soft water optimised BLM.

Model Validation. The field waters selected for performing toxicity tests represented a range of geochemistry conditions recorded in Australia, and provide a comprehensive representation of at least one of the end members for the three key water chemistry parameters, pH, Ca and DOC.

Nickel toxicity was highest in the very soft Magela Creek water for *M. splendida splendida*, *H. viridissima*, and *Chlorella* sp. (Table 3). *Ceriodaphnia dubia* could not be reliably tested in this water due to unacceptable control performance (following USEPA 2002). Water hardness for Magela Creek was 3.5 mg $CaCO_3$/L, which was below the optimal range for this species (Lassier et al. 2006). Acclimation of *C. dubia* to low hardness water did improve control performance for this species, but both survival and reproduction remained below the quality control criteria (USEPA 2002). Both *C. dubia* and *L. aequinoctialis* were most sensitive in Peechelba water, which had high pH, low DOC, and low hardness.

Results of the BLM predictions of EC50 values for each species using the *P. subcapiata* BLM generally showed a tendency to be better at the high effect level (EC50), compared to the low effect level (EC10, shown in Figure 2a), suggesting that this model is able to describe the trends in toxicity to the test species (with the exception of *L. aequinoctialis*), but that additional uncertainty was caused by variability in test results at the low effect level. This is not uncommon and is generally considered to be acceptable where the slope of the concentration-response curve, and therefore also the relative difference between EC10 and EC50 values, is not affected by bioavailability (Peters et al. 2011).

The soft water optimised *P. subcapiata* BLM provided the best fit to the EC50 data for all species except for *L. aequinoctialis*. The relative standard deviation of the error in the BLM predictions was 11.8% for *H. viridissima*, 35.6% for *M. splendida splendida*, 39.8% for *C. dubia*, 41.8% for *Chlorella* sp. The other BLMs defined for other species differ in the way that they model the response to changes in pH. These models do not follow the mechanistic

approach employed by the *P. subcapitata* BLM as this was unable to describe the response of these species over a sufficient range of pH conditions. These models combine a single binding site where Ni interacts competitively with Ca and Mg with a log-linear pH effect and are considered to be valid for Ca concentrations between 2 and 88 mg/L. A soft water optimised *C. dubia* BLM provided the best fit for the *L. aequinoctialis* data with an rsd of 122%.

The soft-water-optimised *P. subcapitata* BLM provided good predictions of nickel toxicity to four of the five tested species, with the majority of test results predicted to be within a factor of two of the observed EC50 result, and within a factor of 3 of the EC10 result (Fig 2 b). All of the test species except *M. splendida splendida* showed a large reduction in intraspecies variability to nickel following normalisation. The intraspecies variability for *M. splendida splendida* was the lowest of the five species before bioavailability normalisation, and bioavailability normalization did not further reduce the overall variation in toxicity.

Discussion

Geochemistry of Australian Waters. The minimum required set of parameters for performing NiBLM calculations (Peters et al. 2011) available from Australian monitoring databases is limited. The spatial coverage of the dataset was limited considering the area of land over which the models are proposed to be applied, and was particularly limited in the west of the continent. Despite this limitation, the surface waters included in the water chemistry dataset showed broad distributions of the parameters known to influence nickel bioavailability, namely pH, DOC, and hardness. Australian surface waters tend to have slightly higher magnesium concentrations, relative to calcium concentrations, than are typically observed in European (Peters et al. 2011). Where measured concentrations of Mg are not available estimates could be based on the relationship in Figure 1 for modelling purposes.

312 **Model Performance in Very Soft Waters.** The parameters for calcium and magnesium
313 binding in all of the NiBLMs currently applied in Europe (Deleebeeck et al. 2009, Deleebeeck
314 et al. 2008, Schamphelaere et al. 2006, Deleebeeck et al. 2007) were estimated from toxicity
315 test data based on a linear relationship between the critical endpoint, such as the EC10 or
316 EC50 expressed as the free metal ion activity, and the activity of the relevant competing ion
317 in univariate sets of tests, where only the parameter of interest was varied (Heijerick et al.
318 2002). These relationships did not perform well at very low hardness when applied to the *H.*
319 *viridissima data*, and tended to overestimate the ECx when compared to the test data. When
320 the test waters were separated into harder (hardness >5 mg $CaCO_3$/L) and softer (hardness
321 <5 mg $CaCO_3$/L) waters, the linear regressions between cation activities and the Ni^{2+} activity
322 at the EC50 values showed steeper slopes for the four softer waters than were observed for
323 the four harder waters (Table 4). This affects the values of the stability constants which would
324 be derived for the binding of Ca and Mg to the biotic ligand.

325 These observations are consistent with previous observations for relationships between
326 hardness and nickel toxicity in very soft, low DOC, waters (Kozlova et al. 2009, Deleebeeck et
327 al. 2007b), where a greater ameliorating effect of hardness on nickel toxicity tended to be
328 found for organisms that normally live in soft waters rather than in hard waters. This effect
329 was observed in previous studies for both acute (Kozlova et al. 2009) and chronic (Deleebeeck
330 et al. 2007b) effects.

331 In the present study, separate sets of univariate tests were not used for calcium and
332 magnesium, and the same log K values for binding to the biotic ligand were assumed for both
333 calcium and magnesium, because of the apparent limitations of the linear interpolation
334 approach for deriving binding constants when the resulting model was applied to very soft
335 water conditions (described earlier). The slopes and intercepts from the linear regression
336 approach used to derive binding constants for the NiBLM for calcium and magnesium, based

337 on the relationship between the Ni^{2+} activity at the EC50 and the Ca^{2+} or Mg^{2+} activity in the
338 test medium, vary depending on the range of water hardness covered (Table 4). This suggests
339 that an alternative approach towards the estimation of binding constant values may be
340 required as the values of the binding constants are conditional upon the water chemistry.
341 Given that the competitive effects of calcium and magnesium become limited at higher
342 concentrations (Deleebeeck et al. 2008), binding constants which vary according to the
343 concentration of the binding ion may be more appropriate than defining discrete ranges of
344 application for various fixed binding constants.

345 Predictions of nickel toxicity to *H. viridissima* in very soft waters by the four different species
346 models were similar because the pH was constant throughout all of the tests (pH 6.5±0.1).
347 Likewise, DOC was absent in these tests. Any difference in the predicted toxicity of nickel
348 among the different models was due to differences in the competitive effects of calcium and
349 magnesium at the biotic ligand between the different models. The *P. subcapitata* BLM was the
350 only one of the existing models that used different binding constants for both calcium and
351 magnesium. As this model did not provide an appreciably better fit to the test data than the
352 models for invertebrates and vertebrates, all of which assume very similar binding constants
353 for both calcium and magnesium to the biotic ligand, this suggested that using the same
354 binding constants for both calcium and magnesium was appropriate for *H. viridissima*.

355 The influence of increasing the competitive effect of calcium and magnesium on nickel toxicity
356 in the BLM has been achieved through an increase in the stability constants for calcium and
357 magnesium binding to the biotic ligand. The root mean squared error (rmse) describes the
358 overall error in the model predictions of the toxicity data. For *H. viridissima*, the minimum
359 rmse was found at a value of log K_{BL} for calcium and magnesium of 5.0, indicating that the
360 best model fit to these data will be obtained with the log K values for binding of calcium and
361 magnesium at the biotic ligand increased from values of 2.0 and 3.3 respectively to a value

362 of 5.0 for both metals. The optimised value of the binding constant for nickel to the biotic
363 ligand of 5.0 was higher than the value of 4.7 derived by Deleebeek et al. (2007b) for
364 cladocerans in water hardness 6 to 16 mg $CaCO_3$/L, although the lowest water hardness in
365 the tests was also lower than that of the field-collected waters used by Deleebeek et al.
366 (2007b), where the minimum hardness was 4.7 mg $CaCO_3$/L, with calcium and magnesium
367 concentrations of 1.2 and 0.4 mg/L, respectively. It is likely that the lower hardness of the
368 softest waters included in the present study resulted in the requirement for higher binding
369 constants to be used for calcium and magnesium.

370 There is an apparent variation in the binding affinities for Ca and Mg which varies with their
371 exposure concentrations. This effect could be interpreted as the existence of a range of
372 binding affinities for Ca and Mg at the biological interface, but with all sites having the same
373 affinity for Ni. Higher affinity sites becoming more important at lower Ca and Mg
374 concentrations would mean that Ni would compete less effectively for binding at the biotic
375 ligand. The changes which have been made to the binding affinities in the models have been
376 inferred through the effect of Ca and Mg on Ni toxicity, and the same outcome could potentially
377 be achieved by relative changes to the affinities of either Ni or Ca and Mg for the biological
378 interface. Given the lack of mechanistic understanding an empirical approach, similar to that
379 used for pH in the Ni BLMs (Nys et al. 2016), may be the most appropriate approach for future
380 modelling efforts.

381

382 **Model Validation.** The existing NiBLMs currently applied in Europe can be applied reliably to
383 Australian systems with calcium concentrations as low as 2 mg/L, which is equivalent to a
384 total hardness of approximately 7 mg $CaCO_3$/L. However, the models performed poorly when
385 applied to waters with a hardness below this level. The soft-water-optimised version of the
386 NiBLM for *P. subcapitata*, using increased values of log K_{BL} for calcium from 2.0 to 5.0 and for

magnesium from 3.3 to 5.0, can be applied in waters with a total hardness as low as 1 mg $CaCO_3$/L. The model has been developed from tests in waters with calcium concentrations up to 10 mg/L. A similarly modified suite of models, all using increased values of log K_{BL} for calcium and magnesium of 5.0, improved the fit of the Australian ecotoxicity data (Figure 3b).

Validation studies have generally aimed for predictions to be within a factor of two of the observed result, although this approach has most commonly been applied to median effect levels from acute studies (Meyer et al. 2018). The increase in uncertainty which is associated with the use of lower effect levels and longer duration studies suggests that the majority of data falling within a factor of three is acceptable for low effect levels from chronic tests (Figure 3d).

The tests with *M. splendida splendida* suggest a slightly more limited range of bioavailability response compared to the other tested species. The EC50 results range over a factor of 4.7 from the most to the least sensitive waters, which compares to a factor of 8.6 and 10.9 for *Chlorella* sp. and *H. viridissima* respectively. The test protocol uses only 5 individuals per treatment level for animal and is based on an imbalance endpoint for ethical reasons. It is possible that the reduced number of individuals used reduces the precision of the tests relative to the plant and invertebrate tests.

The soft-water-optimised *P. subcapitata* BLM always provided the best fit to the EC50 data in the validation tests, except for tests with *L. aequinoctialis* where a similarly modified version of the *C. dubia* BLM provided the best predictions of EC50 data. All individual species models are able to describe the observed bioavailability responses at the EC50 level reasonably well following modification of the log K_{BL} values for calcium and magnesium to 5.0. This is not surprising given the high degree of similarity between the different models and the limited variation in pH amongst the test waters. The *C. dubia* BLM provided the best fit for *L. aequinoctialis* although the performance of all of the BLMs for this species was relatively poor.

412 The principal difference among the species-specific models is the manner in which they
413 describe the effects of pH on nickel toxicity. Recent studies have shown that pH can be an
414 extremely important factor in nickel toxicity, and that sensitivity can be increased at high pH
415 (7).

416 The natural waters used in the present study limited the potential to investigate pH effects
417 due to the relatively limited range of pH conditions represented in the Australian waters. Due
418 to the limited differences observed between different species-specific models in this study a
419 more simplistic bioavailability-normalization approach may be achieved compared to assigning
420 BLMs according to the trophic level of individual species within the ecotoxicity database (i.e.,
421 use the *P. subcapitata* BLM for plants and algae, and so on), by applying more consistent
422 models to all species regardless of trophic level.

423 The adaptations made to the BLMs provided a marginal improvement in the predictions of
424 nickel toxicity to *L. aequinoctialis* in the test waters. Despite showing increasing sensitivity
425 with increasing pH, it would appear that there are additional sources of uncertainty in the *L.*
426 *aequinoctialis* results, although it is also possible that the majority of the remaining variation
427 results from variability in the biological response rather than any consistent effect of water
428 chemistry factors. Testing on another *Lemna* species (Schlekat et al. 2010) found that good
429 predictions were made using BLMs which included calcium competition for nickel binding at
430 the biotic ligand.

431 Uncertainty in the responses for the studies on *L. aequinoctialis* hindered efforts to identify
432 the most appropriate model for this species based on the present study alone. The *C. dubia*
433 BLM provided good predictions for nickel toxicity to *L. minor*, and the soft-water-optimised *C.*
434 *dubia* BLM provided the best predictions for *L. aequinoctialis*. Given that the *C. dubia* BLMs
435 most closely followed the observed pH responses in *L. aequinoctialis*, it is recommended that

436 the soft-water-optimised *C. dubia* BLM be used for predictions of nickel toxicity to this species
437 in soft waters (hardness typically below 10 mg $CaCO_3$/L, upper limit 50 mg $CaCO_3$/L).

438 Due to the similarity among the different species-specific bioavailability models, the
439 differences in terms of model performance for each individual test species were relatively
440 modest. Consequently, the soft-water-optimised *P. subcapitata* BLM has been applied to all
441 species except *L. aequinoctialis*. This suggests that there may be some scope for consolidation
442 of the suite of individual species models which are currently applied in Europe without losing
443 predictive capability.

444 The development tests on *H. viridissima* covered a range of calcium concentrations between
445 0.05 and 10 mg/L for the development studies and 0.25 to 11 mg/L for the validation studies.
446 This represents a range of water hardness between approximately 1 and 50 mg $CaCO_3$/L.
447 There is clearly some overlap in the application ranges of the two versions of the NiBLM, and
448 it would appear that moderately soft surface waters, with a hardness between approximately
449 10 and 50 mg $CaCO_3$/L could be adequately covered by either model. A review of the available
450 ecotoxicity data that fall within the range of hardness values covered by both models is
451 recommended to identify the most appropriate approach to dealing with this transition.
452 Binding constants which vary according to the concentration of the binding ion may be a more
453 appropriate ultimate goal than defining discrete ranges of application for various fixed binding
454 constants.

455 In the absence of such a review, the recommended approach is to apply the bioavailability
456 normalisation approach that is most representative of the water quality at the site of interest,
457 or, in a regional context, able to cover the largest proportion of the waters requiring
458 assessment. The suite of soft-water-optimised NiBLMs would be applied to sites or regions
459 where the water hardness is rarely above 50 mg $CaCO_3$/L, and the models used in the current
460 European NiBLM (Nys et al. 2016) would be applied to sites or regions where the water

461 hardness is rarely below 10 mg $CaCO_3$/L. In applying the two models in this way, the same
462 bioavailability normalization approach is intended to be applied to the entire dataset with
463 differences only in the binding constants for calcium and magnesium at the biotic ligand.

464 Three independent studies (Kozlova et al. 2009, Deleebeeck et al. 2007b, and this work)
465 including acute and chronic endpoints for a variety of different species from three different
466 continents have now shown an increased effect of competing ions on nickel toxicity under
467 very soft water, very low DOC conditions. This evidence suggests that the differences in cation
468 competition observed in very soft waters are related to the water chemistry as all test species
469 were affected similarly, rather than being specific to the physiology of particular soft-water
470 resident species. This work suggests that the mechanisms which govern nickel bioavailability
471 to freshwater organisms exhibit considerable similarity over large spatial scales, and that
472 modified versions of the NiBLMs can potentially be applied throughout a much more diverse
473 range of freshwater systems than previously anticipated.

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Tables

TABLE 1. Key water chemistry parameters for the test waters

Site	pH	Ca	Mg	DOC
		mg/L	mg/L	mg/L
Tea Tree	7.3	2.1	4.7	10.0
Woronora	7.2	2.5	3.5	4.0
Peechelba	8.2	3.1	2.9	0.5
Wellington	7.9	11	7.4	5.0
Magela Creek	6.4	0.25	0.25	3.0

TABLE 2. The sample numbers and percentiles from frequency distributions of key freshwater chemistry parameters in Australia

Parameter	pH	Ca	Mg	DOC
		mg/L	mg/L	mg/L
Number of samples	6418	2622	2653	5196
10^{th} Percentile	5.9	0.4	0.5	1.9
25^{th} Percentile	6.4	0.8	0.9	2.3
50^{th} Percentile	6.9	2.2	1.8	3.8
75^{th} Percentile	7.4	7.0	5.0	8.1
90^{th} Percentile	7.6	45.0	30.0	12.1

561 **TABLE 3. ECx values for nickel toxicity test species in the test waters**

Site	*M. splendida splendida*	*C. dubia*	*H. viridissima*	*L. aequinoctialis*	*Chlorella* sp.
	µg/L (95% confidence interval in parentheses)				
EC50					
Tea Tree	116 (70-163)	20.1	506 (445-554)	221 (192-245)	2,410 (2,140-2,640)
Woronora	72 (57-91)	6.7 (4.7-9.6)	418 (381-454)	19.3 (17-23)	663 (372-1070)
Peechelba	96 (58-140)	4.4 (3.4-5.7)	246 (184-294)	7.8 (5.7-11)	434 (358-474)
Wellington	185 (106-255)	11.8	810 (987-973)	13.5 (10-17)	853 (697-987)
Magela Creek	39 (31-49)	*Not valid*	75 (68-79)	96 (84-119)	282 (212-351)
EC10					
Tea Tree	35 (10-60)	3.6 (0.2-7.2)	210 (132-306)	151 (25-175)	900 (566-1250)
Woronora	30 (18-40)	6.2 (6.2-6.2)	175 (97-217)	13.3 (12-14)	122 (120-124)
Peechelba	30 (6.3-52)	2.0 (0.7-3.9)	35 (15-48)	3.5 (1.0-5.5)	148
Wellington	77 (19-126)	4.9 (2.0-7.0)	55 (36-131)	6.4 (1.8-9.6)	278
Magela Creek	10 (2.2-41)	*Not valid*	32 (17-43)	58 (17-63)	59 (37-89)

562

563 **TABLE 4. Slopes, and intercepts from linear regression analysis of harder and softer**

564 **waters separately from testing with *Hydra viridissima***

Metal	Hardness	Slope	Intercept
Mg	soft	0.031	1.0E-07
Mg	hard	0.020	2.0E-06
Ca	soft	0.045	2.0E-07
Ca	hard	0.026	1.0E-06

565

566

567

Figures

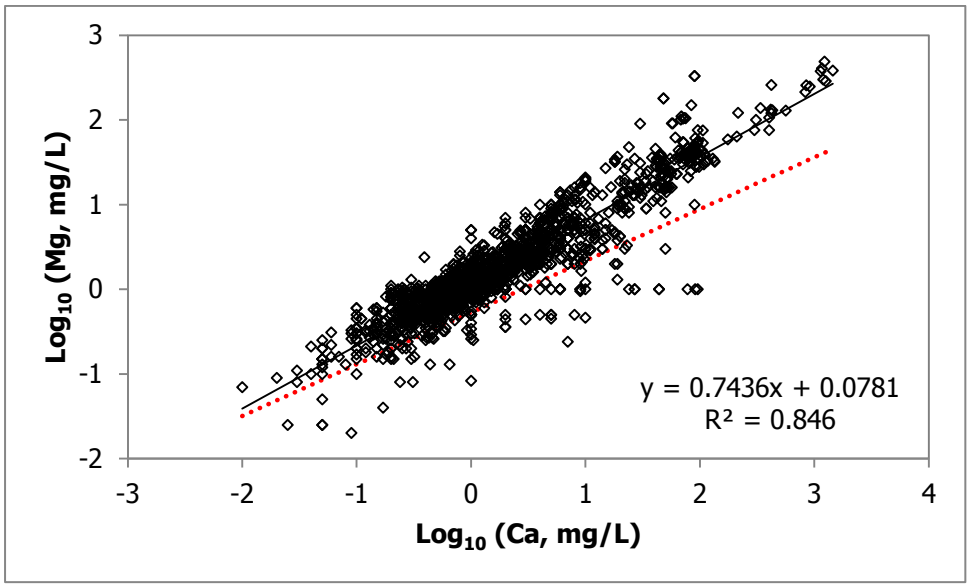
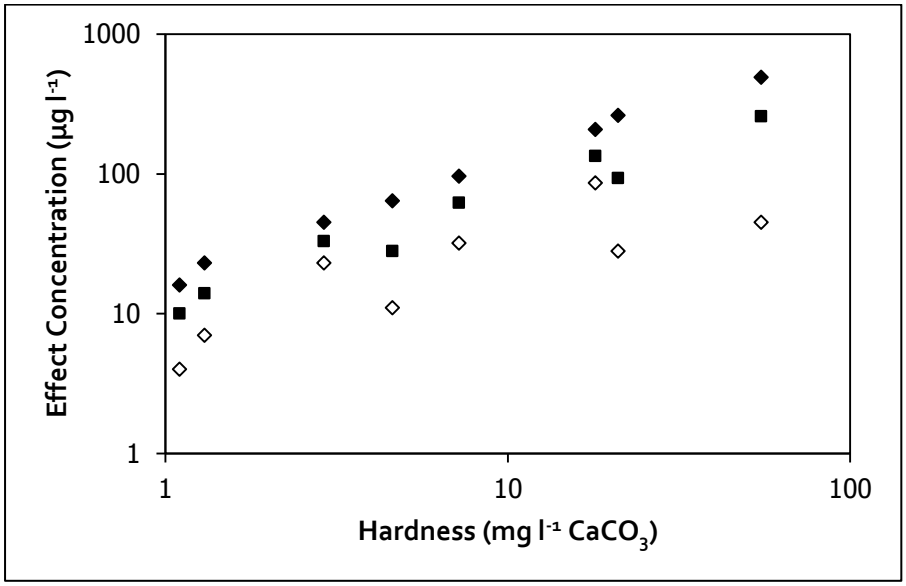


FIGURE 1. Relationship between calcium and magnesium concentrations in Australian waters and the relationship for European waters shown as a red dotted line (11)

573



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575 **FIGURE 2. Results of Ni toxicity tests with Hydra viridissima in synthetic soft**

576 **water. (EC50 solid diamonds, EC25 squares, EC10 open diamonds).**

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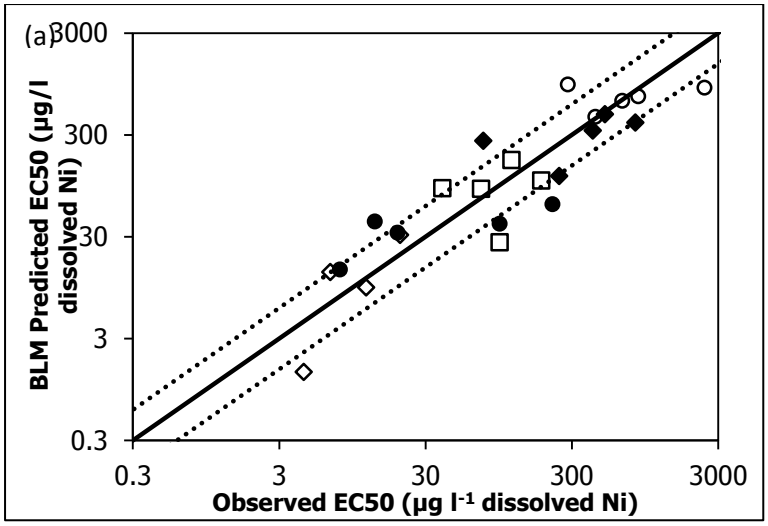
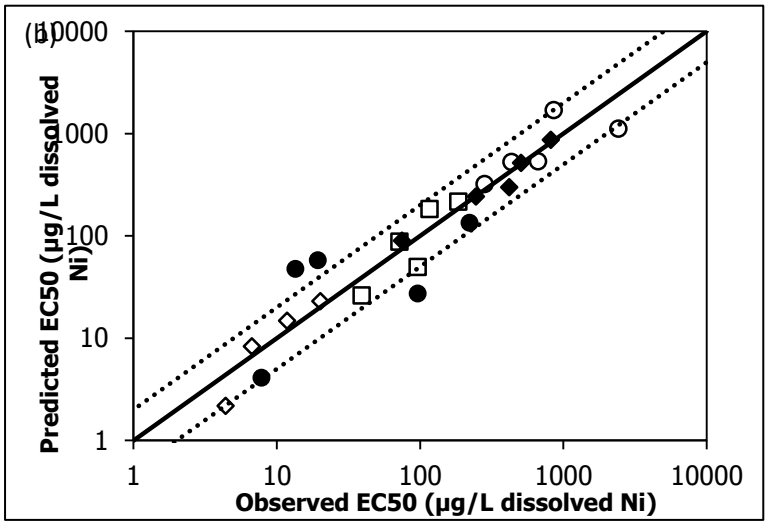

(a)
BLM Predicted EC50 (µg/l dissolved Ni)
3000
300
30
3
0.3
0.3
3
30
300
3000
Observed EC50 (µg l-1 dissolved Ni)

578


(b)
Predicted EC50 (µg/L dissolved Ni)
10000
1000
100
10
1
1
10
100
1000
10000
Observed EC50 (µg/L dissolved Ni)

579

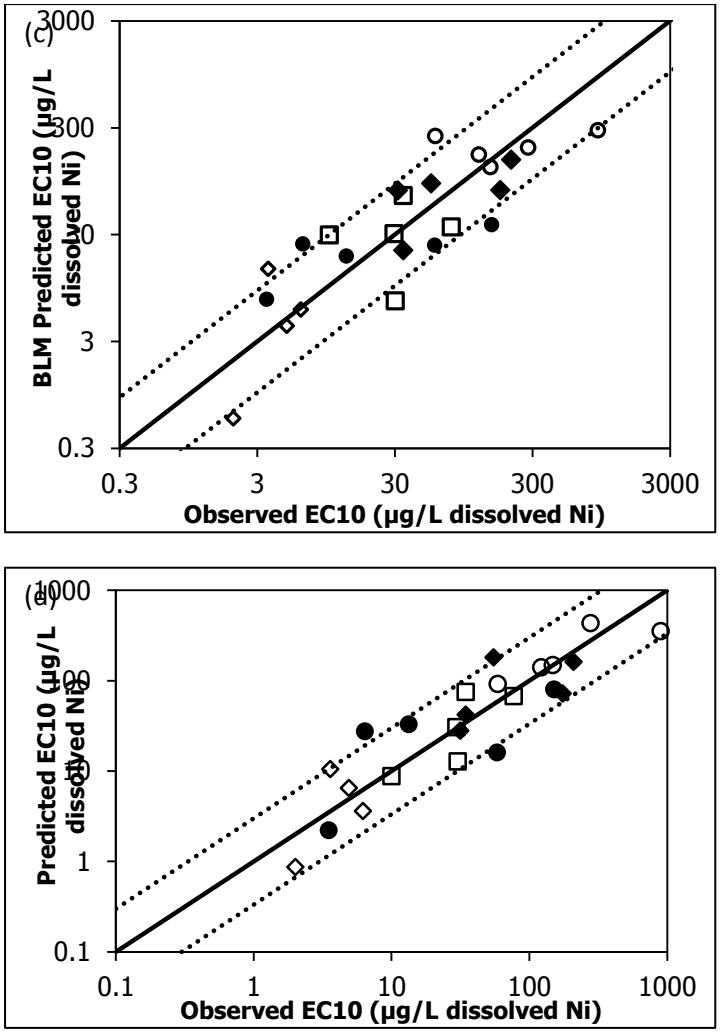


FIGURE 3. **Comparison of observed and predicted Ni toxicity (µg/L dissolved Ni) for (a) EC50 values using the original BLMs, (b) EC50 values using the soft-water-optimised BLMs, (c) EC10 values using the original BLMs, (d) EC10 values using the soft-water-optimised BLMs, for *M. splendida splendida* (open squares), *C. dubia* (open diamonds), *H. viridissima* (filled diamonds), *L. aequinoctialis* (filled circles), and *Chlorella* sp. (open circles) in five Australian test waters, where the solid lines indicate perfect agreement between measurements and models, and the dotted lines indicate a factor of 2 from the observed result for EC50 data, and a factor of 3 from the observed result for EC10 data.**